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A complete mutation screen of the ADPKD genes by DHPLC

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Background. Genetic analysis is a useful diagnostic tool in autosomal dominant polycystic kidney disease (ADPKD), especially when imaging results are equivocal. However, molecular diagnostics by direct mutation screening has proved difficult in this disorder due to genetic and allelic heterogeneity and complexity of the major locus, *PKD1*.

Methods. A protocol was developed to specifically amplify the exons of *PKD1* and *PKD2* from genomic DNA as 150 to 450 bp amplicons. These fragments were analyzed by the technique of denaturing high-performance liquid chromatography (DHPLC) using a Wave Fragment Analysis System (Transgenomics) to detect base-pair changes throughout both genes. DHPLC-detected changes were characterized by sequencing.

Results. Cost effective and sensitive mutation screening of the entire coding regions of *PKD1* and *PKD2* by DHPLC was optimized. All base-pair mutations to these genes that we previously characterized were detected as an altered DHPLC profile. To assess this method for routine diagnostic use, samples from a cohort of 45 genetically uncharacterized ADPKD patients were analyzed. Twenty-nine definite mutations were detected, 26 *PKD1*, 3 *PKD2* and a further five possible missense mutations were characterized leading to a maximal detection rate of 76%. A high level of polymorphism of *PKD1* also was detected, with 71 different changes defined. The reproducibility of the DHPLC profile enabled the recognition of many common polymorphisms without the necessity for re-sequencing.

Conclusions. DHPLC has been demonstrated to be an efficient and effective means for gene-based molecular diagnosis of ADPKD. Differentiating missense mutations and polymorphisms remains a challenge, but family-based segregation analysis is helpful.

Autosomal dominant polycystic kidney disease (ADPKD) is a common genetic disease of the kidney (frequency 1/1000) accounting for approximately 5% of end-stage renal disease (ESRD) in Western countries. The disease is progressive with cyst development and enlargement resulting in bilateral polycystic kidneys and ESRD, typically in late middle age. ADPKD is genetically heterogeneous with two genes, *PKD1* (chromosome region 16p13.3) and *PKD2* (4q21) identified and characterized, plus evidence of rare unlinked families [1–5]. PKD1 accounts for approximately 85% of cases and is associated with a more severe disease course (average age at onset of ESRD of 53 compared to 69 years for PKD2 [6]). The *PKD1* and *PKD2* genes encode related proteins, polycystin-1 and -2, respectively, which probably form part of a receptor/channel complex involved in regulating intracellular Ca^{2+} levels [7].

Presymptomatic diagnosis of ADPKD is possible by various imaging methods (abdominal ultrasound, computer tomography and magnetic resonance) and is relatively reliable in adult patients, especially in the more severe PKD1. However, there is a role for genetic diagnosis in this disorder, especially in patients with equivocal imaging results, those with a negative family history and in cases where a definite diagnosis is required in younger individuals, such as for living related kidney donors. Genetic diagnosis by linkage analysis is possible but is of limited utility due to the requirement for a relatively large family with multiple diagnosed members, to determine the gene involved.

Mutation analysis would be a more direct method of genetic diagnosis and allow genotype/phenotype correlations to be studied. However, mutation screening has proven uniquely difficult in ADPKD. The *PKD1* transcript is large, encoded by 46 exons, and embedded in a complex duplicated genomic area [1, 2]. The 5′ region of *PKD1*, from upstream of exon 1 to exon 33, is reiterated at least five times further proximally on the same chromosome. As this reiteration is relatively recent in evolutionary time, only approximately 2% sequence divergence exists between the *PKD1*-like pseudogenes (*HG* loci) and *PKD1*, complicating specific amplification of the disease gene [8]. In addition to these structural problems, the genetic heterogeneity and marked allelic

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heterogeneity at *PKD1* and *PKD2*, with most mutations unique to a single family [9, 10], have further complicated direct mutation based genetic diagnosis. Although, several screens of the entire approximately 3kb coding region of *PKD2* have been described [10–12], only limited analyses of the duplicated part of *PKD1* have been published. These studies have used methods of long-polymerase chain reaction (PCR), with primers anchored in single copy DNA, and *PKD1* specific-PCR, exploiting the rare differences between the *PKD1* and *HG* loci, to analyze the duplicated region [9, 13–19]. To date, only one screen of the entire *PKD1* gene has been described, showing a mutation detection rate of approximately 50% [9]. However, the methods used in that study, including the use of mRNA derived template and the protein truncation test, are such that the strategy is not readily adapted for routine diagnostic needs.

Recently, a new semi-automated method has been introduced for mutation analysis in human genes, denaturing high-performance liquid chromatography (DHPLC) [20, 21]. This system is based on the differential adsorption of homo- and hetero-duplexes to a hydrophobic matrix in a chromatographic column. Amplicons with a mismatch have decreased interaction with the matrix and are thus eluted earlier than the normal amplicon, resulting in an alteration of the elution profile. This method has a high level of sensitivity and specifically, is semi-automated and does not require analysis by electrophoresis. Due to the advantages of this technique it has now become the method of choice for screening large and multiexon genes involved in human genetic disease [22].

In the current study, we describe a mutation screen of the entire *PKD1* and *PKD2* genes by DHPLC using genomic DNA from a cohort of 45 ADPKD patients. An overall detection rate of approximately 70% was achieved. We propose this approach as an efficient and effective way to perform molecular diagnostics in ADPKD.

METHODS

Selection of the cohort

Forty-five unrelated ADPKD probands were selected for screening. Informed consent was obtained from each participant and the study was approved by the appropriate IRB and/or Ethics Committee. This cohort consisted of typically sized families that had not previously been subjected to linkage analysis. All probands in the study met established ultrasound criteria for ADPKD diagnosis [23]. A blood sample for DNA isolation was drawn from each proband and from available family members interested in participating.

DNA isolation and field inversion gel electrophoresis (FIGE)

Genomic DNA was extracted by standard phenol-chloroform procedures. An aliquot of DNA (5 μg) was digested with EcoRI and separated by FIGE using the FIGE Mapper Electrophoresis System (Bio-Rad, Herts, UK). Gels were Southern blotted and hybridized with the *PKD1* IVS1 probe CW10, as previously described [1].

PCR amplification and generation of DHPLC amplicons

The duplicated region of *PKD1* was amplified as five PCR fragments that were either anchored in single copy DNA or mismatched with *HG* sequence (details are in the **Results** section and Table 1). Long-PCR fragments (>3kb) were amplified as previously outlined [9] using the rTth DNA polymerase (PE Applied Biosystems) in the supplied DMSO containing buffer. A combination of Amplitaq (PE Applied Biosystems, Foster City, CA, USA) and Taq Extender (Stratagene, Heidelberg, Germany) was used for the GC-rich exons 1 of *PKD1* and *PKD2* [9]. Each amplicon for DHPLC analysis was amplified as a fragment of 150 to 450 bp using Amplitaq and the supplied (non-DMSO) buffer for *PKD2* and a dimethyl sulfoxide (DMSO) containing buffer for *PKD1* (detailed in Tables 2 and 3 and [9, 24]). The primers were positioned approximately 20 to 30 bp from the intron-exon boundary to allow the detection of splicing defects but minimize intronic polymorphism. Due to the presence of a frequent intronic deletion (IVS9+28del7, found in 8 of 46 samples), a second reverse primer (9 int), positioned only 2 bp from the intron/exon boundary, was used to screen samples carrying the deletion polymorphism. The availability of a known mutation of this splice site (IVS9 +1G→T) was used to show the utility of the closer primer.

Heteroduplexes of amplicons were generated by heating the PCR products at 95°C for three minutes, cooling 0.1°C per second to 65°C, incubating at 65°C for 30 minutes, cooling 0.1°C per second to 37°C, with a final incubation of 10 minutes.

DHPLC and gradient generation

Denaturing high-pressure liquid chromatography was performed using the Wave system (Transgenomic Inc., Omaha, NE, USA). Crude PCR product, sufficient to generate an elution peak of 3 to 5mV (200 to 500 ng, in Buffer C), was injected into a preheated, fully equilibrated chromatographic column (C18 reverse-phase, non-porous polystyrene-divinyl benzene matrix; DNASep Column; Trangenomic Inc.). A linear gradient of 5% triethylammonium acetate (TEAA) (Buffer A) and 25% acetonitrile + 5% TEAA (Buffer B) with a flow rate of 0.9 mL/min was generated for each amplicon. Each DHPLC run included a DNA loading step (5% drop for loading of Buffer B), a linear separation gradient (2% Buffer B slope per min, 4.5 min), a wash step (75% acetonitrile; Buffer D, 0.5 to 1 min) and an equilibration step (0.9 to 1.2 min). When DMSO buffer was used in the PCR reaction, longer wash and equilibration steps were re-

Table 1. PKD1 specific fragments

Fragment	Primers (5′-3′)	Annealing temperatures °C	Position[a] (nt)	Size bp	Exons
Gen 1	[9]	64	2033–4310	2278	1
Gen 2–12	CCAGCTCTCTGTCTACTCACCTCCGCATC CCACGGTTACGTTGTAGTTCACGGTGACG	68	17,647–26,372	8726	2–12
Gen 13–15	[17]	70	26,246–30,631	4386	13–15
Gen 15–21	[17]	68	30,603–33,980	3378	15–21
Gen 22–34	CCGTGTAGAGAGGAGGGCGTGTGCAAGGA TCGGCAAGGACCTGCTGGATCAGGTCTTC	70	36,883–44,383	7501	22–34

[a] Genomic DNA (Accession No. L39891)

quired. Total run times were therefore 9.4 minutes and 8.6 minutes for the *PKD1* and *PKD2* amplicons, respectively.

Fragment melting profile analysis and optimization of DHPLC conditions

The melting profile of each fragment was analyzed using the Wavemaker version 4.0.32 (Transgenomic Inc.) software. The process by which the melting point is determined is based on the Fixman and Friere implementation of Poland's algorythmn [25], which calculates the probability that a base is in the helical duplex form or the nonhelical, single-stranded form.

DHPLC conditions for each amplicon were optimized by use of the available positive controls (details are in Tables 2 and 3). Each fragment was eluted at the predicted melting temperature, and plus and minus 1 and 2°C. If multiple domains were predicted by the Wavemaker software, then a wider range of temperatures was tested and the final analysis performed under two sets of conditions (see Tables 2 and 3 for the final conditions for each fragment). A set of eight random samples, plus the positive control, were used for each optimization process and conditions were adjusted so that the DNA eluted from the column at, or greater than, four minutes. Resolution was optimized so that the elution profile of the positive control differed most from the normal sample. When no positive control was available, conditions were considered to be optimal at the temperature and corresponding gradient immediately before a significant decrease in the retention time of the amplicon was observed and/or an excessive broadening of the peak, indicating excess denaturation. This temperature was typically located in the range of 50 to 75% helical fraction for the amplicons analyzed.

DNA sequencing

Samples showing an aberrant elution profile were reamplified and subjected to direct sequencing using the Big-Dye Terminator kit (PE Applied Biosystems). The PCR product was purified by polyethylene glycol 8000 (PEG) and ethanol precipitation and resuspended in water. An aliquot of the product (30 to 180 ng) was combined with each primer (3.2 pmol) and DMS0 (1 μL) for sequencing reactions, analyzed on an ABI377 sequencing machine and assembled using Sequencher 3.1.1 software.

Validation of mutations

To analyze the segregation in families of characterized mutations the DHPLC elution profiles of all family members (always including two affected cases from different generations) were compared. Due to the reproducibility of the elution profile, the mutant genotype was assigned to samples resembling the mutant profile. When more than one sequence change was present in the same fragment, validation was performed by direct sequencing of all samples. Missense mutations were screened on 146 normal chromosomes by DHPLC.

RESULTS

DHPLC analysis of the ADPKD genes

A method was developed to screen the entire *PKD1* and *PKD2* genes from genomic DNA by DHPLC. The duplication of the *PKD1* genomic region (exons 1 to 33) required that first-round long-PCR reactions were necessary to specifically amplify these exons. This was achieved for the regions bordering single copy DNA by employing a primer anchored in single copy DNA; the method used to amplify exon 1 and exons 22 to 34 has previously been described (Table 1 and Fig. 1) [9]. For the rest of the duplicated area, specific amplification of *PKD1* was obtained by employing primers that matched differences between *PKD1* and *HG* sequences. Two previously described primer pairs exploiting a number of mismatches in exon 15 were used to amplify exons 13 to 21 [17]. To analyze exons 2 to 12, one primer was positioned in the region of IVS1 that is deleted in most copies of the *HG* [8] and the second in exon 13, which is not present in the IVS1-containing *HG* locus (manuscript in preparation). The specificity of these products was determined by amplification of somatic cell hybrids

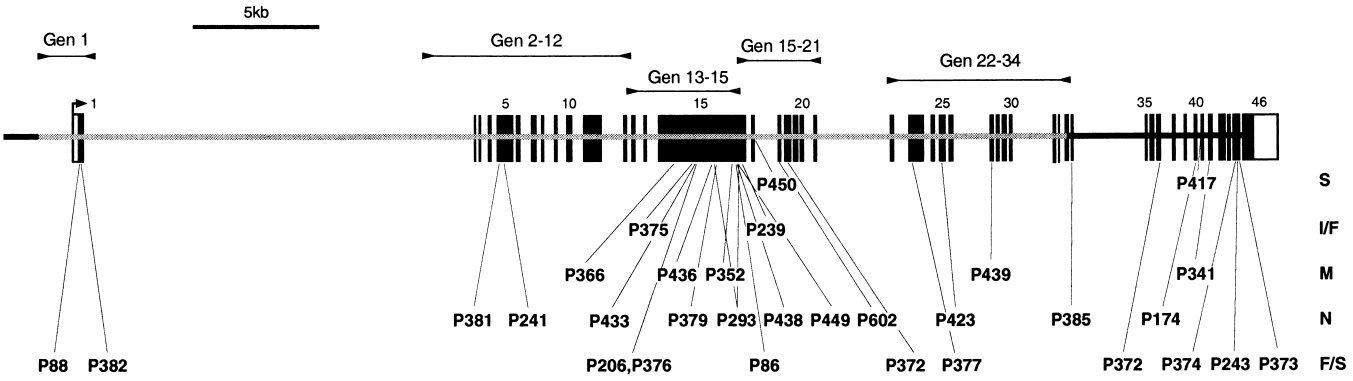

Fig. 1. Map of the *PKD1* gene showing the fragments employed for specific amplification of the duplicated part of the gene (top) and the locations of detected mutations in the indicated pedigrees (bottom; details are in Table 4). The mutation types are: I/F, in-frame deletion; M, missense; N, nonsense; F/S, frame-shifting deletion/insertion; and S, splicing.

containing either just *PKD1* (radiation hybrid Hy145.19) or only the *HG* loci (P-MWH2A) [1].

All exons were amplified as fragments of 150 to 450 bp, a size range found to be ideal for DHPLC analysis (Tables 2 and 3). For the duplicated region, the *PKD1*-specific first-round product was used as the template, while for the remainder of *PKD1* and for *PKD2*, DHPLC amplicons were amplified directly from genomic DNA. Large exons were split into more than one fragment (exon 15 was analyzed as 13 overlapping fragments) and intronic primers located 25 to 30 bp from the intron/exon boundaries. The 15 *PKD2* exons were amplified using previously described primer pairs and conditions (Table 3) [24]. In total *PKD1* was amplified as 64 fragments and *PKD2* as 17, making a total of 81 to analyze both genes.

The temperatures and initial Buffer B concentration of the elution gradient for DHPLC analysis were determined for each of the amplicons using the Wavemaker software and empirically (**Methods** section; Tables 2 and 3). Analysis temperatures for *PKD1* were generally higher than for *PKD2*, reflecting the higher GC content. If the melting profile of the fragment was reasonably homogenous one analysis temperature was used. However, for 14 *PKD1* and 8 *PKD2* fragments, two different melting domains were detected and, in these cases, the amplicon was analyzed under two different sets of conditions (Tables 2 and 3). An important determinant for establishing ideal DHPLC conditions was the availability of positive controls. Previously characterized mutations or polymorphisms were available as positive controls for 58/64 *PKD1* and 15/17 *PKD2* amplicons (Tables 2 and 3). These controls included a variety of changes, but the most subtle change (a substitution) was selected if available. As well as helping to determine ideal analysis conditions, the positive controls also allowed the quality of analysis to be monitored by inclusion with each run of the fragment. The analysis of positive controls also permitted the sensitivity of the method to be assessed. All of our previously described base-pair mutations and polymorphisms [9] were detected with the amplicons and analysis conditions described in our current study, indicating that DHPLC is a very sensitive method for detecting base-pair changes.

Mutation screening by DHPLC in a cohort of ADPKD patients

To determine the achievable detection rate, a cohort of 45 uncharacterized ADPKD patients was analyzed. Initial screening of *PKD1* for large-scale rearrangements by FIGE did not identify any mutations. The entire coding regions of both the *PKD1* and *PKD2* genes (or until a clear mutation was identified) were screened by DHPLC. A total of 266 aberrant profiles were detected and characterized, illustrating the variable nature of the *PKD1* gene. In the 32 patients in whom both genes were fully screened, an average of 5.4 DNA changes (range 0 to 14) were found per patient. These changes were characterized by sequencing, and segregation with the disease was analyzed when samples from other family members were available. Figures 2 and 3A show examples of aberrant DHPLC profiles from analyses of the *PKD1* and *PKD2* genes and examples of sequencing are shown in Figure 4. The way in which the reproducibility of the DHPLC profile was used to trace the mutation within a family is illustrated in Figure 3B.

Of the total changes, 29 were definite mutations, 26 from *PKD1* and 3 from *PKD2*, consisting of 13 nonsense mutations, 12 truncating deletions and/or insertions, a large and a small in-frame deletion and 2 splicing defects (Table 4). One of these mutations, 5225delAG (a previously described mutation; [15]) was found in two families, while two nonsense mutations, Q1838X and S2164X were present in the same patient and found to be inher-

Table 2. Details of PKD1 amplicons

Exonic fragment	Size *bp*	PCR annealing temp. °C	Primers		DHPLC conditions		Positive control
			Forward (5′-3′)	Reverse (5′-3′)	Temp. °C	Initial % buffer B	
1a	220	64	CCTGAGCTGCGGCCTCCG	CAGTTGACGCGGCAGGCG	72	51	L13Q
1b	180	66	TGCGAGCCCCCCTGCCTC	AACCCGCCCACGCCCGCCCGTCC	70	49	P61L
2+3	340	62	TAGGGGCTCTGGCCCTGAC	CCAGCCAGGACCCCACCCAAAG	54, 64	56, 53	S75F
4	266	61	CATAGACCCTTCCCACCAG	CCTGGCTGGGAAGGACAGA	64, 66	53	IVS4+21delC
5a	262	62	TGGAGCCAGGAGGAGCAGAA	CAGAGGGACAGGCAGGCAAA	64, 66	53, 51	S225X
5b	303	64	AGCCCTCCAGTGCCTCCTTT	GCACGGCCGTCACGTGATAG	66	54	Q266X
5c	310	63	TGGGACTTCGGAGACGGCT	GAGTGGGCAGCAGACACTCA	66	55	1330C/T
6	280	62	ACCGTTGACACCCTCGTTCC	TCTCTGCCCCAGTGCTTCAG	64, 66	55	1420C/T
7	329	62	CTAACCACAGCCAGCGTCTC	CTGTGAGGGTGGGAGGATGG	67	55	IVS7+1G→A
8	226	64	GCGGCTCGGTCCCCAGTCT	GGAGGGCAGGTTGTAGAACGTG	66	52	P/S572
9ext	295	62	GGAGTCTGGGCTTCAGGCTG	CTGGGAACCACTCTGGTGGC	64	52	IVS9+1G→T
9int	228	62	GGAGTCTGGGCTTCAGGCTG	CACCCACCACCCAGAGTCCC	64	52	IVS9+1G→T
10a	184	62	GGCCTGTGGGCAAATCAGGG	TGGGGGTGGCAGGAGGCGTC	64	49	IVS9-4A/G
10b	201	61	AGGGGGACGCTGGTGCCCTG	GGGAACAGACCCAGGTCAGG	66	50	2296delC
11a	425	63	GTCCACGGGCCATGACCG	CCAGCCACTGGGGAGACCAC	65	57	Q705X
11b	257	62	GGCAGAGGTGGGCAATGG	AGCCGGGCACGAAGGTGGC	66	53	None
11c	326	62	GTGTCAGCGCCCGCTTTG	CTGTGTGAGCACCCTGTCTGC	66	55	2905A/C
12	201	61	CTCCACAGAGCCCAGGCTG	ATCCCCTCCCCTCCCCACCC	62	50	IVS11-5C/T
13	244	60	CTGCCACCTGGGCTCACTG	TGCCCACCCCAAACCGGC	66	53	3274C/T
14	198	60	CTCACTGCGTCCCACCGC	CTGAAAGGCAGTGGCCCC	65	50	M/T1092
15a	382	63	TGGGGAGCAGGTGGGGGTGC	AGACGCGCACATCCGCCTGGGCCG	64	56	P/S1168
15b	363	63	CGTGCGCCTGGAGGTCAAC	GGCTGCGTGGGGATGCAG	68	56	None
15c	373	61	CGTGCTGGTCTTCGTCCTGG	TGTAGCGGTAGGGGAACGG	64	56	4291delG
15d	380	61	GTTTGTGCAGCTCGGGGAC	AAGCGTGGGTGACCTCCG	65	56	4474C/T
15e	376	61	CCCGCCAGCTACCTGTGG	GCGGAGCCCACCTCGTTC	65, 66	54, 53	E1537X
15f	378	61	CTTCCGCTCCGTGGGCAC	GGAGGCGGCCACCATCAG	65	56	Q1636X
15g	374	63	AGCGCCTGGGCCGACTGCAC	AGCTGCCCCCAAAAGGGC	65	56	5518C/T+5584C/T
15h	363	63	GAGCCCGGAGGCAGCTTC	GGGAGCACCTCGGGGTTG	66	56	5893C/T
15i	377	62	AGCTGTCACCTTCCGCCTG	GCACCTGGATCTCCAACAGCC	67	53	F1992L,1993delT
15l	340	62	GCTGGTCATCCTGTCGGGC	CACCAGGTTGGAGGCGTTC	66	57	Y2092C
15m	200	60	CCAGGGCCGAGCACTCCTAC	GTCAACGTGGGCCTCCAAGT	67	50	Q2158X
15n	360	62	AGCGCAACTACTTGGAGGCCC	TGGGGTCGTAGGACTCGCTC	65, 66	56	C2229X
15p	196	60	CGCCTGGTGCCCATCATTG	GGACGGGTGAGGGGCATG	65, 66	50, 48	None
16	230	63	AGGCCACGTCGCCCCTTG	GAGGCTGGGCTGTCCAAGG	67	52	IVS16+1G→C
17	203	60	GAGGTAACCCCACTCCCACG	ATCCCCAGCCCGCCCACAC	65	50	7376C/T
18	353	63	AGAGGGTTGCGCCCCCTC	ATCCCGCTGCTCCCCCCACGCAGG	66, 67	56	R2430X
19	286	62	TCCCGTGATGCCGTGGGG	CAGGTGGCAGTCTCGGGG	67, 68	54, 52	None
20	260	62	CCACCTGCTCACCACCCC	GCAGGGGTACAGGTCTTGGTCCC	66	53	IVS20+47G/T
21	226	60	GCGCTGCTGACAGCTTGC	ATGCGGGGCAGGGTGAGC	66	52	H/R2638
22	220	62	AGTGGGGCCAGGAGCGGG	GGGCGGGTGGCATGGGGC	66	51	T/M2708
23a	349	62	CCCTCCCTCTACCTCCCTGTC	CACTGAGGTTGGCCAGGGC	64	55	A2752D
23b	431	62	GGGCCTGGCTGCCACTTC	AAGGCCAGGGGGCCGCGTG	64, 67	57, 55	G2858S
24	222	61	CAGGCGTGTGACCTGCGC	TGCCCTGCCCTGCCAGCTG	65	52	S/L2958
25	313	61	CTGGGCTCACGTCCGCTAC	GCTCCCAGGAGCACAGGGTC	66	53	E3020X
26	289	62	GAGAAGGCACAGCTTGCACG	AGAGCAGGGGAGGCCCTG	65	54	9541C/T
27	240	62	GCAGACCGAGCCTCCCAC	AGGGGCAGAGCTTGGCAG	65	52	W3180X
28	217	61	TGCGAGCCTGACCTCCCTC	CCAACCTCCCACGGAGTGG	65	51	9880G/A
29	316	62	TTGGGCAGGGTGGTCCTG	GGAAGGGCTGGGCAGGAAG	65, 66	55, 53	None

(Continued)

Table 2. (Continued)

Exonic fragment	Size *bp*	PCR annealing temp. °C	Primers		DHPLC conditions		Positive control
			Forward (5′-3′)	Reverse (5′-3′)	Temp. °C	Initial % buffer B	
30	202	61	CAGCCTCACCTGTGTGGCC	TCCATTCCCAGTACTCCCGG	65	50	None
31+32	338	62	GAGCAGGTCTGAGCTGCCG	GCACCAGGGCTCGAGGTTTC	62, 66	55, 53	Q3394X
33	286	62	GGGTGGGCTGTGTGTGTGAC	GCAAGGGTGAGCTTCAGAGCC	65	54	R/Q3434
34	154	59	GCCCACCCTATGCCTCCTG	AATCCCCCCTCCCCCGAGAGCCGG	62	47	Q3473X
35	219	60	GGGCTTGGCTGCAACTGC	GGAGGGGCTAGGGGCATC	64	51	Q3513X
36	280	63	CCTCCCTGTGAGCTGCCTCTC	GGCCTGTAGCCTACCCCTGG	65, 66	54, 52	10943InsT
37	325	61	TCCATCACGGGGGACCCCTCT	AAAGGGGGACAGGAGTGTCCT	65	55	E3631D
38	268	60	AAAGCCCTGCTGTCACTGTGG	TAGGGTCTGGCTGGACTAAAG	65	53	IVS38+13A/G
39	265	61	GGGTCTCTGGTGGCCGCTCA	ATGCCAGAGCTCCGCTAAAGG	66	53	IVS39+1G→C
40	265	61	CACTCCTGTTGGGTTTTTGATG	CGGCACTCCTGGAGAACTACT	65	53	Y3780X
41	330	64	CGGCCTCCTGACCAGCCTGGCTC	TAGGCCAGCGGGGGGCCGGAGGAGTG	66	54	Y3818X
42	319	60	TGCCACCCGCTCCTACTGA	TGGAGGCGCGGGGTCT	70	53	11554C/T
43	378	64	CGTCCCTCCCGCCCTCCTGA	TCTGTCTGCTTGCAGCCCTGGGGTGTG	68	56	12169C/T
44	285	62	GCCTCGCTGCTCTTCCTG	GCTGAGCTGAGCTAAGACGCCCTCC	64, 66	54	R4020X
45	379	61	AGCTCAGCTGTACGCCCTCA	TGTCCCTCTCCCCCCCACTG	65	56	V/I4145
46a	450	72	GAGAGGGACACGCCCTGGGCTCTGC	GGCAAGGCGGCTGGGCAGTGCTGG	66	57	12838C/T
46b	240	68	CCCGTGGCCCATCCCCGGGCCTGCGG	TACGTGCAGCCATTCTGCCTGGCCC	66	52	13135G/A

Table 3. Details of PKD2 amplicons

Exonic fragment	Size *bp*	DHPLC conditions		Positive controls
		Temp. °C	Initial % buffer B	
1a	279	69, 71	54	R28P
1b	291	67, 69	54	None
1c	220	69	51	IVS1+1G→C
2	188	56, 57	50, 48	W201X
3	268	57, 60	54, 52	V262M
4	365	60	56	R361X
5	362	57	56	G381R
6	298	58	54	1417delC
7	318	51, 57	55, 53	Q555X
8	292	56, 58	54	1782delC
9	217	55	51	IVS9+1G→C
10	216	56	50	2075insCG
11	235	57	52	2125delC
12	195	59	50	Y762X
13	235	60	52	E814X
14	248	61, 63	52	R872X
15	382	58, 60	56	None

ited on the same chromosome from the affected father. In this case it is not clear if both mutations occurred simultaneously or if a second mutation occurred on an already mutant allele. In total, definite mutations were identified in 29 of 45 pedigrees (64%). Samples were available to show segregation with the disease in 16 of these pedigrees.

A second group of probable mutations were defined, consisting of non-conservative missense changes in which segregation with the disease was demonstrated. These changes were not found on 146 normal, unrelated chromosomes or previously described as a polymorphism. Two mutations fitted this category, Y2092C, a change in the 16th PKD repeat and L3851P a previously described [26] substitution in the 3rd extracellular loop in the transmembrane region. Leucine 3851 is a highly similar valine in polycystin-1 orthologs in mouse and Fugu fish, while tyrosine 2092 is conserved in the C-β strand of many PKD repeats [27] and also found at this position in the mouse, although it is a cysteine in Fugu [28]. Two further non-conservative missense changes: R1340W (PKD repeat 7 and conserved as a positively charged histidine in mouse and Fugu) and E1811K (PKD repeat 12, conserved as a negatively charged glutamic acid or aspartic acid in mouse and Fugu) were characterized. Neither of these changes was found in the cohort of normal individuals or has previously been described, however, due to the lack of available samples, segregation could not be tested. Finally, a conservative change, I3167F, which has not been found on normal chromosomes, but where segregation could not be demonstrated, was identified. This residue is an isoleucine in mouse and Fugu and lies within the possible lipid binding PLAT domain [29]. Although it is difficult to prove if these substitutions are mutations, it is worth noting that the entire coding region

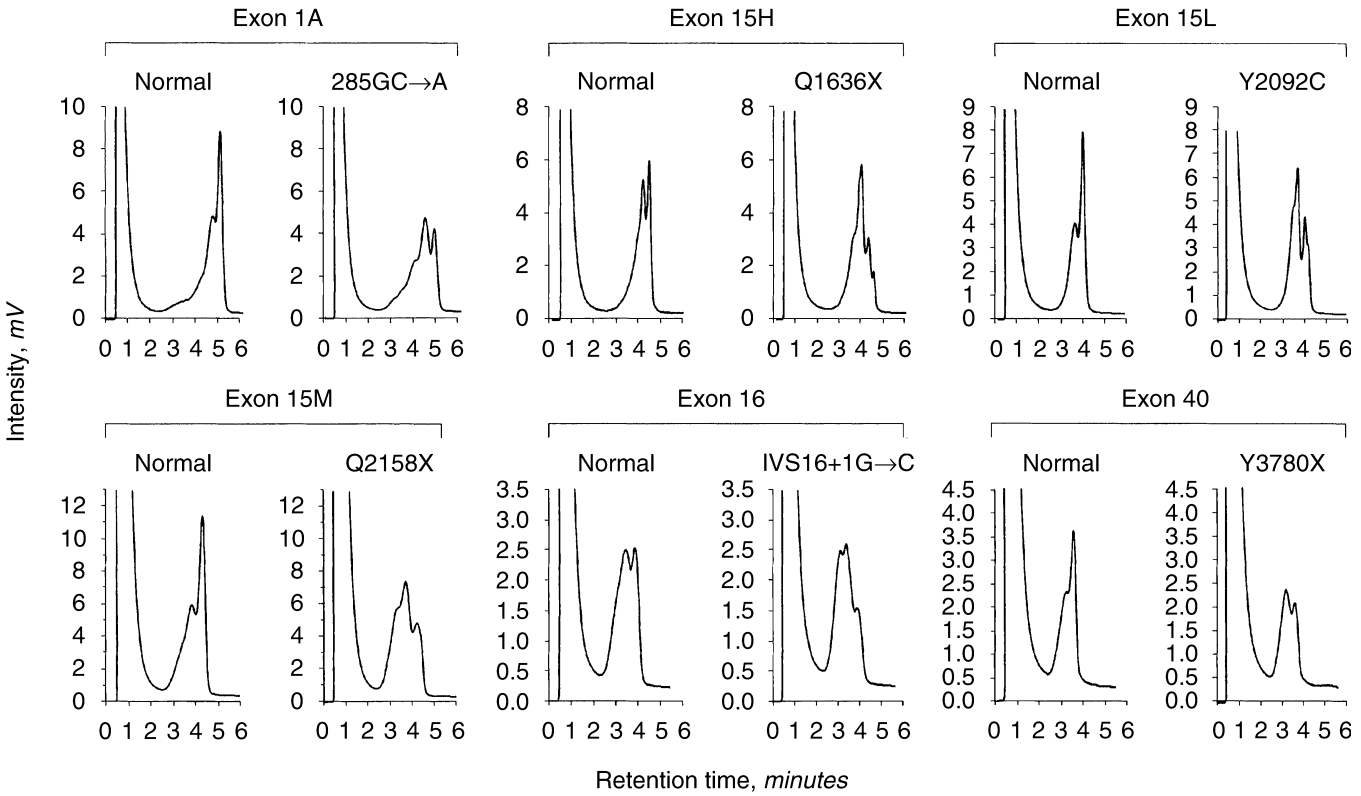

Fig. 2. Examples of aberrant denaturing high-pressure liquid chromatography (DHPLC) profiles obtained from analysis of *PKD1* exon fragments, as indicated.

of *PKD1* and *PKD2* was screened and these were the only likely pathogenic changes detected. Inclusion of the segregating missense changes increases the overall detection rate to 69%, while inclusion of all the possible missense substitutions shows a mutation detection rate of 76%.

In addition to the mutations, and reflecting the frequency of DHPLC profile changes, a high level of polymorphism was detected. Seventy-one different polymorphic changes to *PKD1* and two *PKD2* polymorphisms were detected (Table 5). Thirty-four of these have been described in previous studies, leaving 53% as newly characterized changes. The changes were defined as polymorphisms because they were intronic changes beyond the splice consensus sites ($N = 14$), in the 3′ UTR ($N = 1$), silent exonic changes (not altering an amino acid) ($N = 35$) or amino acid substitutions that did not segregate with the disease in this or previous studies ($N = 21$). These polymorphisms are clearly a complication to mutation analysis in *PKD1*. However, because of the reproducible nature of the DHPLC profile (demonstrated by the segregation analysis; Fig. 3B), frequent polymorphisms, once identified by sequencing, were recognized in subsequent samples by DHPLC "signature-based" genotyping. A total of 54 (~20%) DHPLC false positives were detected in the screen of 45 patients, in which no sequence change was identified. This, however, probably overestimates the final false positive rate that decreased as optimal analysis of each fragment was achieved during the study.

DISCUSSION

This study describes the first screen of the entire *PKD1* and *PKD2* genes for mutation. A largely genetically uncharacterized cohort of ADPKD patients was analyzed, similar to that which may be seen in a routine diagnostic setting. The method employed, DHPLC, has proven suitable for rapid detection of base pair changes with an overall detection rate of approximately 70%.

DHPLC is becoming the method of choice for analyzing large multi-exon genes, such as those causing cystic fibrosis and tuberous sclerosis [30–32], because of the semi-automation of the analysis, the high level of sensitivity (100% of the known mutations in this study) and the cost of analysis, calculated at ~$1 per sample run [31]. While direct sequencing may be considered the best means to analyze small genes (with few exons) [33], larger more complex genes benefit from a screening approach, such as DHPLC, to flag abnormal exons, which thus decreases the number of exons to be sequenced, and hence the total cost of the analysis. This consideration is of particular importance when selecting a method for

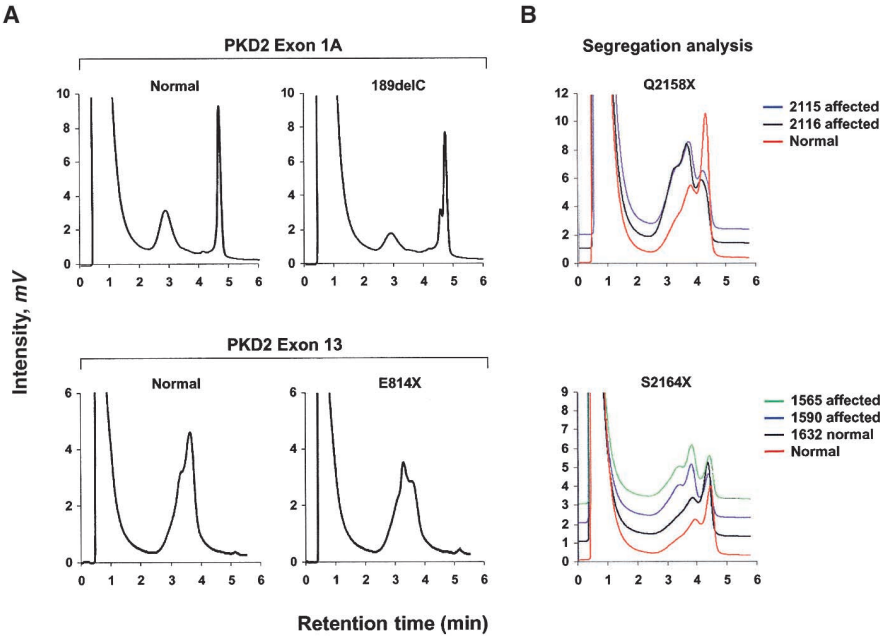

Fig. 3. (*A*). Examples of aberrant DHPLC obtained from analysis of *PKD2* exon fragments, as indicated. (*B*; top) Segregation analysis of the mutation Q2158X; found in the proband (2116) and affected father (2115) but not normal control and (bottom) S2164X; present in the proband (1565) and her affected father (1590), but not unaffected brother (1652) or control.

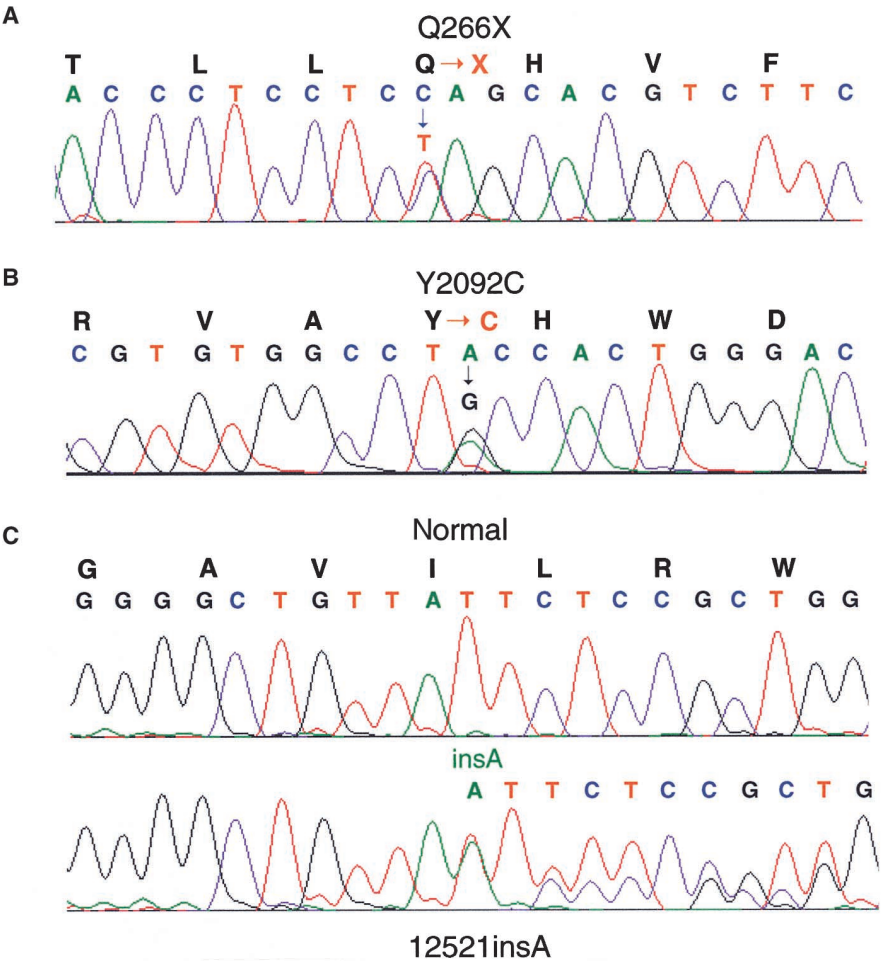

Fig. 4. Examples of sequence data showing: (*A*) nonsense mutation Q266X; 1007C→T; (*B*) missense change Y2092C; 6486A→G and (*C*) insertion, 12521insA.

Table 4. Details of mutations

Mutation designation	Exon fragment	Amino acid[a]/ DNA change	Pedigree	Segregation[b] demonstrated	Reference
PKD1					
285GC→A	1a	24↓	88	Yes	Novel
319insCC	1a	36↓	382	NP	Novel
C230X	5a	901C→A	381	NP	Novel
Q266X	5c	1007C→T	241	NP	Novel
R1340W	15c	4229C→T	366	NP	Novel
4804del252	15e	Δ1532-1615	375	NP	Novel
Q1636X	15f	5117C→T	433	Yes	Novel
5225delAG	15f	1671↓	206,376	Yes; NP	[15]
E1811K	15g	5642G→A	436	NP	Novel
Q1828X	15h	5693C→A	293	Yes	Novel
W1837X	15h	5722G→A	379	NP	Novel
Y2092C	15l	6486A→G	352	Yes	Novel
6651del28	15m	2144↓	86	Yes	[9, 16]
Q2158X	15m	6683C→T	438	Yes	Novel
R2163X	15m	6698C→T	449	Yes	[9]
S2164X	15m	6702C→G	293	Yes	Novel
I2260del	15n	6989del3	239	Yes	Novel
IVS16+1G→C	16	Δ2306-2355	450	Yes	Novel
Q2376X	17	7337C→T	602	NP	Novel
7596del10	18	2461↓	372	NP	Novel
8531delC	23a	2773↓	377	Yes	Novel
Y2991X	25	9184C→G	423	Yes	Novel
I3167F	27	9710A→T	439	NP	Novel
Q3473X	34	10628C→T	385	NP	Novel
11192delC	37	3660↓	386	NP	Novel
Y3780X	40	11551C→G	174	Yes	Novel
IVS40-1G→A	41	Δ3804-3845	417	NP	Novel
L3851P	42	11763T→C	341	Yes	[26]
12474delGG	45	4087↓	374	NP	Novel
12521insA	45	4101↓	243	NP	Novel
12617delC	45	4135↓	373	NP	[9]
PKD2					
189delC	1a	63↓	353	Yes	Novel
2073insCG	10	691↓	338	Yes	Novel
E814X	13	2440G→T	283	Yes	Novel

[a] Symbols are: ↓, truncated after; Δ, amino acids deleted (inclusive)
[b] NP, segregation analysis not possible because of lack of family members or available samples

routine molecular diagnostics where high throughput and cost considerations are important.

The results of this screen have illustrated the sensitivity of DHPLC with all previous changes detected and the majority of new changes being simple substitutions. The position of the change within the amplicon did not influence the detection rate, as in some other conformation sensitive systems (such as SSCA and gel-based heteroduplex analysis [34]), with substitutions detected immediately adjacent to the primer (for example, see exon 9 in the **Methods** section). This allowed the use of primers just 20 to 30 bp from the intron/exon boundaries, minimizing the detection of intronic polymorphisms. Initial analysis of the single copy exons of *PKD1* with previously described primers located 40 to 60 bp from the splice sites [35] showed an unacceptably high level of variant profiles due to intronic changes. Our screen of the ADPKD genes also has allowed the optimal size of amplicons to be analyzed. Previous studies have suggested optimal sizes for DHPLC analysis of 150 to 700 bp [22], however, we found a significant decrease in detection rate (using positive controls) and an increased rate of false positives in fragments greater than 450 bp. Consequently, although larger fragments would have been beneficial to minimize the number of analyses for large exons (like exon 15), amplicons of no larger than 450 bp were employed.

Conditions for DHPLC were determined using the Wavemaker 4.0.32 software, but for some regions were also calculated employing a Stanford University Web based site, Stanford Melt program (http://insertion.stanford.edu/melt.html). Although the predictions were generally similar, for some amplicons the WM4.0.32 program predicted significantly higher temperatures and in general we had greatest success with these calculations. For a few fragments, neither prediction was accurate and in these cases the availability of positive controls was invaluable for establishing optimal analysis conditions.

A particular problem that was previously evident in mutation screens of *PKD1*, but emphasized in this study of the entire gene for base-pair changes, is the level of polymorphism [9, 16, 36]. In cases where the entire gene

Table 5. Description of polymorphisms

Designation	cDNA change or amino acid position	Location	Frequency %	Reference
P/L61	393C/T	EX1	4	Novel
1330C/T	L373	EX5	10	[9]
1420C/T	H403	EX6	2	[9]
IVS6+26C/T		IVS6	2	Novel
IVS7-27C/T		IVS7	2	Novel
1921C/T	H570	EX8	4	[9]
P/S572	1925C/T	EX8	2	Novel
IVS9+28del7		IVS9	16	Novel
IVS9-4A/G		IVS9	33	Novel
2320C/T	H703	EX11	2	Novel
2905A/C	A898	EX11	8	[16]
2911G/A	P900	EX11	4	[16]
2941C/T	D910	EX11	4	[16]
3025G/A	T938	EX11	2	Novel
IVS11-5C/T		IVS11	2	Novel
3274C/T	G1021	EX13	14	[16]
3322A/G	L1037	EX13	8	[16]
M/T1092	3486T/C	EX14	8	Novel
3583C/T	A1124	EX15	6	[16]
3586C/T	S1125	EX15	6	[16]
P/S1168	3713C/T	EX15	2	Novel
W/R1399	4406T/C	EX15	4	[16]
4474C/T	A1421	EX15	2	Novel
5047G/A	T1612	EX15	2	Novel
S/L1684	5262C/T	EX15	2	Novel
5383C/T	A1724	EX15	14	[16]
5518C/T	H1769	EX15	2	Novel
5584C/T	N1791	EX15	2	Novel
5665G/A	A1818	EX15	10	Novel
5893C/T	A1894	EX15	2	Novel
5974G/A	L1921	EX15	2	[16]
V/I1943	6038G/A	EX15	2	Novel
6667C/T	D2152	EX15	2	Novel
IVS15+22C/T		IVS15	2	Novel
7138C/T	G2309	EX16	4	[9]
7376T/C	L2389	EX17	25	[15]
7652C/T	L2481	EX18	21	[15]
IVS20+47T/G		IVS20	19	Novel
H/R2638	8024A/G	EX21	8	[15]
P/S2674	8231C/T	EX22	2	[9]
T/M2708	8334C/T	EX22	2	[9]
IVS22+8G/A		IVS22	2	[15]
G/R2814	8650G/A	EX23	6	[9]
8650C/T	L2816	EX23	4	[9]
S/L2958	9084C/T	EX24	2	Novel
T/N2977	9141C/A	EX24	2	Novel
V/M3057	9380G/A	EX25	2	Novel
9541C/T	P3110	EX26	14	[13]
IVS27-13T/C		IVS27	2	Novel
9880G/A	T3223	EX28	2	[13]
10168C/T	S3319	EX30	2	Novel
IVS30-25G/A		IVS30	2	Novel
R/Q3434	10512G/A	EX33	2	Novel
A/V3511	10743C/T	EX35	16	[13]
IVS37+42delTT		IVS37	4	Novel
IVS38+13A/G		IVS38	6	[35]
11554C/T	D3781	EX40	2	Novel
IVS41+5ins3		IVS41	2	Novel
11890C/T	S3893	EX42	4	Novel
12169C/T	A3986	EX43	2	Novel
12184C/G	A3991	EX43	2	[35]
I/V4044	12341A/G	EX44	23	[41]
IVS43-34C/A		IVS43	10	Novel
A/V4058	12384C/T	EX45	2	[41]
12484A/G	A4091	EX45	6	[36]
P/S4123	12578C/T	EX45	2	Novel
12617C/T	L4136	EX45	2	[35]
V/I4145	12645G/A	EX45	2	[42]
S/F4189	12777C/T	EX46	2	Novel
12838C/T	P4209	EX46	23	[13]
13135G/A	(3′ UTR)	EX46	2	Novel
PKD2				
R/P28	83G/C	EX1	5	[12]
IVS3-23G/A		IVS3	25	[11]

was analyzed, an average of 5.4 changes were detected, indicating that the ratio of polymorphic changes to mutation is very high at 4:1 to 5:1. The problem of common polymorphism was overcome to some extent by using the reproducible shape of the DHPLC profile to identify the change without sequencing ("signature-based" genotyping). Furthermore, because of the change specific shape of the profile, we did not find that common polymorphisms masked other mutations. When a mutation and polymorphism were detected in the same fragment a novel profile was generated.

The difference in the rate of polymorphism between *PKD1* and *PKD2* was striking in this study, with only two polymorphisms detected in *PKD2*. Although it is possible that there are special reasons for the level of changes at *PKD1* (such as the polypyrimidine tract in IVS21 [37] or conversion by *HG* changes [14]) the GC rich nature of *PKD1* is probably the major explanation. The GC richness results in a high level of CpG dinucleotides that are known to be hot spots for mutation because the methylated cytosine is susceptible to spontaneous deamination to thymine [38]. Although this change only accounted for 4 of 20 mutation-associated substitutions in *PKD1*, 54% of substitution polymorphisms occurred at CpG dinucleotides. An influence of the unusual IVS21 sequence was not clear, as changes were found throughout the gene and no mutations and only 8 of 70 polymorphisms matched known *HG* sequences. The large size of the *PKD1* transcript also may partly explain the higher level of polymorphism (a target four times greater than *PKD2*).

The high level of polymorphic change at *PKD1* makes it difficult to determine if a missense substitution is a mutation. Tests of whether the change is conservative and if the residue is conserved in polycystin-1 orthologs seems to be of only limited value as some of the polymorphisms also appear to be non-conservative and some are at conserved sites [9]. Segregation with the disease is an important test, as lack of co-inheritance rules out the change as a mutation, as long as the diagnosis in other family members is clear. Therefore, even within a routine molecular diagnostic setting, the availability of at least one sample from another affected family member would be beneficial. Of course, segregation with the disease is not proof that the missense change is a mutation, but hopefully as more molecular screening of *PKD1* is reported, it will be possible to assemble lists of segregating mutations and non-segregating polymorphisms. The difficulties associated with missense changes are, however, likely to remain a complication of analysis at this locus until a functional test for the protein is available.

The uncertainty over the status of many missense changes means that the final mutation detection rate in this study cannot be reported with certainty. The lowest rate, just considering the definite mutations, is 64% and higher than previous studies of *PKD1* alone, partly reflecting the approximately 10% of mutations to the *PKD2* gene detected in this typical ADPKD population. If all the possible missense changes also are considered as mutations, the detection rate rises to 76% and is comparable to comprehensive screens of other large genes associated with disorders with genetic and allelic heterogeneity, such as tuberous sclerosis or Alport syndrome [32, 39]. The undetected changes presumably consist of more subtle splicing defects (caused by changes away from the donor and acceptor splicing sites and by silent exonic changes), promoter mutations and the likelihood of a low level of changes undetected by DHPLC. In addition, larger rearrangements of *PKD2* were not sought and the possibility that some of these cases may be due to as yet unmapped ADPKD genes should be considered.

As in previous studies, mutations were spread throughout the *PKD1* and *PKD2* genes and, except for one case, were unique to a single family within the study [9]. However, with the accumulating data on mutations, it is interesting to see that five changes matched previously described mutations (Table 4). Even if all the putative substitutions are included as mutations the majority of changes (~82%) were clear truncating or substantial in-frame changes, likely to inactivate the gene. This finding is of importance in the diagnostic setting indicating that, despite the problem differentiating missense mutations and neutral polymorphisms, clear results of mutation detection could be reported in 64% of cases. We had previously estimated from sequencing small regions of *PKD1* that missense mutations would represent a higher level of mutations [9], but that has not been borne out by this study. It remains possible, however, that missense mutations in highly conserved regions of the protein will be important sites of mutation. The nature of the detected changes is consistent with cyst development being associated with polycystin loss, through a two-hit mechanism [40], or haploinsufficiency. Despite the evidence that mutations may inactivate, it will be interesting to see if any significant correlations with phenotype are associated with the type and/or position of mutations. Such studies should now be possible with this improved and simplified mutation screening approach.

We have illustrated the possibilities for routine molecular diagnosis in ADPKD by direct mutation detection using DHPLC. Genetic diagnosis is likely to become increasingly important as potential therapies become available, to determine the status of at-risk individuals at an early stage before significant renal damage has occurred.

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